

# Development of a Multiplex CARS Flow Cytometer for Label-Free, Real-Time Classification

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## Abstract:

An effective, label-free method of identifying unknown particles in a flow cytometer is presented through the application of multiplex coherent anti-Stokes Raman spectroscopy (MCARS) to a microfluidic chip. Various designs for microfluidic channels were theoretically modeled using COMSOL Multiphysics and fabricated using photolithography. A program was written in MATLAB to perform principal component analysis in order to determine the spectra of unknown substances and to classify them in real-time. As proof of principle, chemically specific differentiation of polystyrene microbeads and oil was experimentally demonstrated.

## Introduction:

Flow cytometers are used to observe and characterize individual microscopic particles, such as cells, in a stream of fluid. The major advantage of using these devices, as opposed to examining single droplets in a microscope, is the high throughput of sample in a given time frame. Current cytometers use laser scattering to classify cell size and shape, and fluorophores, which bind to specific proteins on the surface of a cell, for chemical identification. However, such endogenous fluorophores can disrupt the physiological systems of living samples. Moreover, it requires advance knowledge of the substances present. Additionally, the large fluorescence bandwidth renders multiplexing of multiple fluorophores challenging; it also requires hardware-intensive structures, including the use of several pump lasers at different wavelengths and unique sets of excitation/emission filters and photomultipliers (PMTs) for each multiplex channel. Hence, there is a need for chemically specific, label-free classification in a flow cytometer.

In this work, MCARS was applied to cytometric analysis on a microfluidic chip. Multiplex coherent anti-Stokes Raman scattering (MCARS) is a label-free method of optical imaging that uses the vibrational chemical signature of molecules to uniquely identify and visualize them. As a result, it eliminates the need for fluorophores. Moreover, the coherent pumping of Raman bands and multiplex detection allow for high sensitivity and full spectrum measurements (Figure 1). This new addition to flow cytometry complements traditional sizing and morphological information with fluorophore-free chemical information. Moreover, the narrow Raman spectral peaks, compared to the broad emissions from fluorophores, further facilitate multiplexing.

The aim of this project was to fabricate a MCARS cytometer that could effectively flow a mixture of various cells in a

stream of fluid, and analyze their chemical signatures in real-time, thereby allowing for down-stream sorting of the sample. (See Figure 1.)

**Microfluidic Design.** Fluid flow is governed by the Navier-Stoke equation. In microfluidics, flow speeds are approximately quartically proportional to the channel diameter. Therefore, microfluidic channels were designed to perform hydrodynamic focusing, a method of maintaining fast and thin sample flows by means of adding a sheath fluid. Such focusing was theoretically modeled in COMSOL Multiphysics through the Navier-Stokes incompressible flow and convection / diffusion modules (Figure 2). It was found through this form of analysis that for a 1000 psi pressure drop across the sample microfluidic channel, a volumetric flow rate of approximately 3 microliters per second could be achieved. This would allow the system to analyze approximately 30 to  $3 \times 10^5$  cells per second, depending on sample concentration

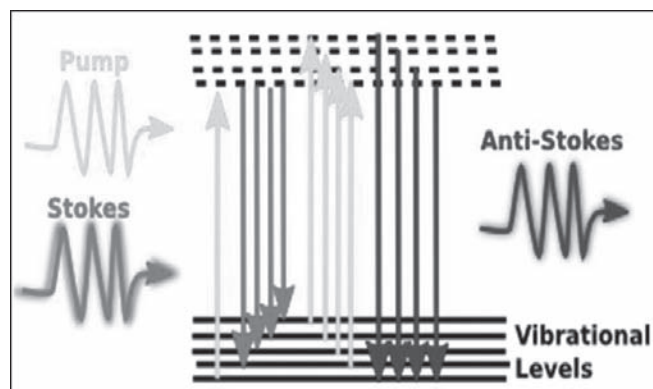


Figure 1: The energy-level diagram for MCARS is shown. The use of white light as the Stokes beam allows gathering of information about all the vibrational energy states of the molecule.

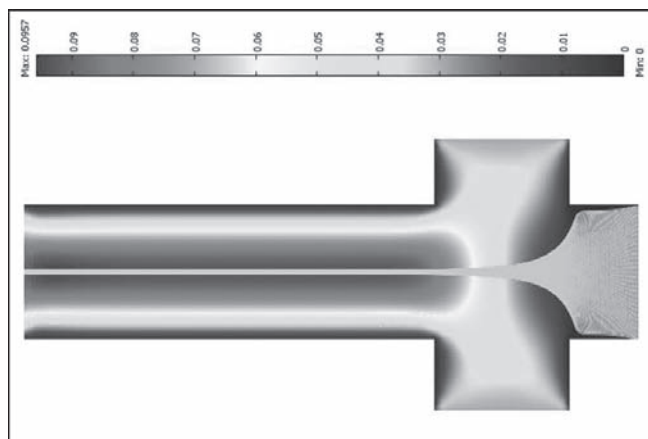


Figure 2: A COMSOL velocity field plot of hydrodynamic focusing is shown. The three inlet channels are all 150  $\mu\text{m}$  wide and the sample particles (represented by the grey streamlines) from the center inlet are focused into a thin stream by the sheath fluid.

and the CCD frame rates, which is comparable to current industry standards. Such a microfluidic channel design was chosen and drawn in Auto CAD, as shown in Figure 2.

**Fabrication.** Fabrication of the microfluidic channels was done through photolithography. A chrome mask of the channel designs was purchased from Photosciences, Inc. First, a 30  $\mu\text{m}$  layer of SU-8 (negative photoresist) was spun on a silicon wafer. A two-step bake was performed to cure the resist. Then, it was exposed to 365 nm UV light for 13 seconds and developed for 5 minutes. The prepared wafer was then coated with 100 nm of chrome and 200 nm of gold using electron-beam evaporation. Next, polydimethylsiloxane (PDMS) was prepared by mixing Sylgard 184 with its curing agent in a 10:1 ratio. The mixture was degassed under vacuum, poured uniformly onto the wafer, and cured for 4 hours at 70°C. Then, the PDMS layer was peeled off and holes were punched in it for attaching nanoports. Finally, the PDMS was treated with oxygen plasma for 10 seconds at 50W before sealing it to a glass slide, completing the fabrication process.

**Analysis.** In order to analyze the MCARS spectral data collected from these microfluidic devices, a chemometric tool called principal component analysis (PCA) was used. PCA is a mathematical method that decomposes data into an alternative basis, maximizing variance between subsequent principal components, or basis vectors. Each substance's spectrum can then be easily identified as linear combinations of the first few principal components.

A GUI was written in MATLAB to perform this analysis. First, it applied a Savitsky-Golay filter and normalized the data. Next, PCA was performed. Then, the projection of each spectra onto the first few principal components was graphed. This caused clustering which allowed one to differentiate and identify various substances. This method was used successfully to differentiate polystyrene beads in oil although the MCARS spectra of both substances are very similar (Figure 3).

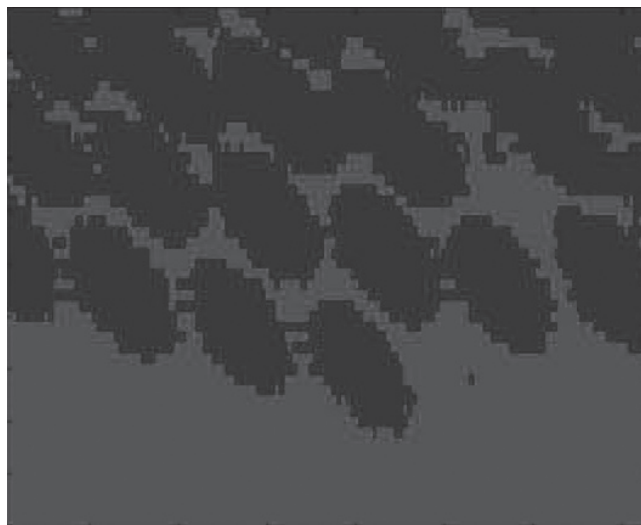


Figure 3: An image of polystyrene beads (black) in oil (grey) is shown. It was reconstructed in MATLAB by performing PCA on the MCARS spectral data collected.

Once the spectrum of each substance is determined through a training set, real-time identification can be implemented by grabbing one spectrum of a particle flowing by and matching it to the various spectra collected beforehand through the training set.

### Conclusions:

Microfluidic channels with the capacity for hydrodynamic focusing were modeled theoretically, designed, and fabricated. A program in MATLAB was written to differentiate and identify various substances flowing through the microfluidic devices by applying PCA to the spectral data collected. In this manner, chemically specific differentiation of polystyrene and oil was experimentally demonstrated. This sets the groundwork for the implementation of a flow cytometer chip that will perform real-time identification of particles using MCARS.

### Future Work:

Once real-time particle identification is accomplished, it will be useful to introduce a sorting device to classify the particles into bins accordingly. Common methods of doing this include the use of micro-actuators, magnetic coatings, or electric potentials. It is also beneficial to introduce cameras in order to determine the size of particles through scattered light.

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